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(21) International Application Number: PCT/US90/01535 (22) International Filing Date: 26 March 1990 (26.03.90) (30) Priority data: 328,999 27 March 1989 (27.03.89) US (71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors: BRENNER, Sydney ; 17B St. Edward's Passage, Cambridge (GB). MILLER, Jeffrey, Allan ; 25 Gulf Road, Derry, NH 03038 (US). (74) Agents: FRANK, George, A. et al.; E.I. du Pont de Nemours and Company, Legal Department, 1007 Market Street, Wilmington, DE 19898 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PROCESS FOR NUCLEIC ACID DETECTION BY BINARY AMPLIFICATION (57) Abstract A nucleic acid assay based on the enzymatic combination of the products of two amplification reactions carried out with two sets of two primers specific for two different regions within the target nucleic acid is provided.		

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TITLE

PROCESS FOR NUCLEIC ACID DETECTION
BY BINARY AMPLIFICATION

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FIELD OF INVENTION

This invention relates to the detection of nucleic acid sequences and more specifically to a process of combining the products from the amplification of two portions of a target nucleic acid sequence.

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BACKGROUND OF THE INVENTION

The development of practical nucleic acid hybridization methods which can be used for detecting nucleic acid sequences of interest has been limited by several factors. These include lack of sensitivity, complexity of procedure, and the desire to convert from radiometric to nonradiometric detection methods. A variety of methods have been investigated for the purpose of increasing the sensitivity nonradiometric procedures. In one general approach, improvements in the total assay procedure have been examined, with concomitant effects on the issues of complexity and nonradiometric detection. In another approach, methods which increase the amount of nucleic acid to be detected by such assays have been pursued.

25 U.S. Patent 4,358,535, issued to Falkow, describes a method of culturing cells to increase their number and thus the amount of nucleic acid of the organism suspected to be present, depositing the sample onto fixed support, and then contacting the sample with a labeled probe, followed by washing the support and detecting the label. One drawback to this method is that without culturing the organism first, the assay does not have adequate sensitivity. Adding a culture step, however, is time consuming and not always
30 successful. Maniatis et al., Molecular Cloning: A

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Laboratory Manual, Cold Spring Harbor Laboratory, pp.390-401 (1982), describe a method in which a nucleic acid of interest is amplified by cloning it into an appropriate host system. Then, when the host organism
5 replicates in culture, the nucleic acid of interest is also replicated. This method also suffers from the requirement to perform a culture step and thus provides for a procedure that is time consuming and complicated.

An alternative approach to increasing the quantity
10 of nucleic acids of organisms has been described in U.S. patents 4,683,202 and 4,683,195. These patents disclose "a process for amplification and detection of any target nucleic acid sequence contained in a nucleic acid or mixture thereof". This process employs an in vitro
15 cycling mechanism which doubles the nucleic acid sequence to be amplified after each cycle is complete. This is carried out by separating the complementary strands of the nucleic acid sequence to be amplified, contacting these strands with excess oligonucleotide
20 primers and extending the primers by enzymatic treatment to form primer extension products that are complementary to the nucleic acid annealed with each primer. The process is then repeated as many times as is necessary. An advantage of this method is that it can rapidly
25 produce large quantities of a small portion of the sequence of the nucleic acid of an organism of interest. A disadvantage of this method is that the detection of the nucleic acids produced, using a direct assay method, is complicated in that the amplification process can
30 produce nucleic acid sequences which are not faithful copies of the original nucleic acid which was to be copied. These erroneous nucleic acid sequences can provide false positives in the assay which increase the background noise and thus decrease the sensitivity of
35 the entire method.

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Numerous DNA probe assays have been described in the past for the detection of nucleic acids of interest. Falkow's method (above) first renders the target nucleic acid single-stranded and then immobilizes it onto a solid support. A labeled probe which is complementary to the target nucleic acid is then brought into contact with the solid support. Any excess probe is washed away and the presence of the label in the resulting hybrid is determined. A disadvantage of this method is that it is time consuming and cumbersome. The assay steps, i.e., hybridization and washing steps are carried out in a sealed pouch which contains the membrane (solid support) as well as the buffer solution.

Hill et al., WO 86/05815, describe a variation of the above assay format employing nitrocellulose coated magnetic particles to which the target DNA is affixed, followed by direct hybridization with a biotinylated probe and detection using a streptavidin-conjugated reporter.

Dunn et al., Cell, Vol. 12, 23-36 (1977), describe a different hybridization format which employs a two-step sandwich assay method employing polynucleotide probes in which the target nucleic acid is mixed with a solution containing a first or capture probe which has been affixed to a solid support. After a period of time, the support is washed and a second or reporter (labeled) probe, also complementary to the target nucleic acid but not to the capture probe, is added and allowed to hybridize with the capture probe - target nucleic acid complex. After washing to remove any unhybridized reporter probe, the presence of the reporter probe, hybridized to the target nucleic acid, is detected.

Ranki et al. U.S. Patent 4,563,419, disclose EPA 0 154 505, W086/03782, and EPA 0 200 113. It is to

be recognized that all of these employ an assay procedure in which the first or capture probe is immobilized onto a solid support prior to hybridization.

A further variation has been described in German Preliminary Published Application 3,546,312 A1. This method, like that described by Ranki et al., employs a capture probe and a reporter probe which hybridize to distinct portions of the target nucleic acid. The target nucleic acid is contacted in solution by the two probes. The first, or capture probe, contains a binding component, such as biotin, that is capable of binding with a receptor component, such as streptavidin, which has been affixed to a solid support. After formation of the capture probe - target nucleic acid - reporter probe complex, a streptavidin-modified solid support is added. Any unhybridized reporter probe is washed away followed by the detection of the label incorporated into the complex bound to the solid support. An advantage of this technique over that disclosed by Ranki et al. is that the hybridization, which takes place in solution, is favored kinetically. Some disadvantages are that the length of the target nucleic acid affects the overall efficiency of the reaction which decreases with increasing target nucleic acid length. Also, sandwich nucleic acid probe assays, whether heterogeneous two-step or one-step, or utilizing solution hybridization, are not as sensitive as the direct assay method.

A disadvantage of all of these techniques relates to the need to employ a specific hybridization step in order to obtain the necessary specificity and thus proper identification of the target nucleic acid sequence of interest.

DISCLOSURE OF THE INVENTION

The nucleic acid assay of this invention for the detection and/or measurement of a preselected nucleic

acid sequence in a sample suspected of including a nucleic acid containing said preselected sequence comprises the steps of:

- 5 (A) rendering the target nucleic acid single-stranded;
- 10 (B) amplifying two specific nucleic acid sequences contained within the preselected nucleic acid sequence, said specific nucleic acid sequences being positioned such that when either sequence is amplified under amplification conditions, the extension product of either sequence cannot serve as a template for the synthesis of the other sequence, by
- 15 (1) treating the strands with two sets of two oligonucleotide primers, one set for each different specific sequence being amplified, under conditions such that for each different sequence being amplified an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said
- 20 sets of primers are selected so as to be sufficiently complementary to the different strands of each specific sequence to hybridize therewith such that the extension products synthesized from one primer from each of the two sets of primers, when separated from their
- 25 respective complements, can serve as templates for the synthesis of the extension products of the other primer from each of the two sets of primers; wherein one of the primers of each set of
- 30 primers contains a LoxP sequence at its
- 35 5'-end;

- (2) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;
- 5 (3) treating the single-stranded molecules generated from step (2) with the two sets of primers of step (1) under conditions that primer extension products are synthesized using each of the single
- 10 strands produced in step (2) as templates; and
- (4) repeating steps (2) and (3) to produce sufficient primer extension products for detection and/or measurement;
- 15 (C) treating the products of the two separate amplification reactions by the recombinase enzyme Cre; and
- (D) detecting and/or measuring that product of
- 20 step (C) resulting from the two separate amplification reactions.

DETAILED DESCRIPTION OF THE INVENTION

The nucleic acid assay of this invention comprises the following overall process for the detection of

25 target nucleic acids of a preselected sequence:

- a) Using the polymerase chain reaction (PCR) nucleic acid amplification method described in U.S. 4,683,202, incorporated herein by reference, two
- 30 specific nucleic acid sequences, within the preselected sequence, are amplified by first annealing the denatured target nucleic acid present in the sample with two sets of oligonucleotide primers complementary to the specific nucleic acid sequences. These primers are designed such that one primer of each set of primers contains a LoxP
- 35 sequence attached to its 5'-end. The LoxP sequence is

defined by a 34 -nucleotide sequence

5'...ATAACTTCGTATAGCATACATTATACGAAGTTAT...3' . [See Sternberg et al., J. Mol. Biol., Volume 197, 197-212 (1986).] The specific nucleic acid sequences are

5 positioned such that when either sequence is amplified under amplification conditions, the extension product of either sequence cannot serve as a template for the synthesis of the other sequence. During amplification, each extension product formed from each set of primers
10 is complementary to one of the two specific nucleic acid sequences within the preselected nucleic acid sequence and is a template for further primer extension. This process is then repeated as necessary in order to produce the desired amount of primer extension products
15 for detection and/or measurement.

b) Adding the recombinase enzyme Cre to the products of the two separate amplification reactions, allowing the enzyme to combine the amplification product from the first amplification with the amplification
20 product from the second amplification. The resulting product is of a length of the combination of the two specific nucleic acid sequences plus 34 additional nucleotides.

c) Detecting the product of the above step by,
25 for example, gel electrophoresis. Such detection can differentiate between the desired combination product on the one hand and the combination products formed from the coupling of each amplification product with itself.

The term "PCR" as used herein in referring to the
30 process of amplifying target nucleic acid sequences employing primer oligonucleotides to produce by enzymatic means a greatly increased number of copies of a small portion of the target nucleic acid is described in U.S. patent 4,683,202.

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The PCR target amplification reaction requires approximately 20 to 30 repeat cycles in order to produce a sufficient quantity of the amplified target nucleic acid for further hybridization. Denaturation of the amplified nucleic acid can be accomplished by treatment with alkali, acid, chaotropic agents, or heat, although the preferred method is to place the amplified target nucleic acid in a boiling water bath for at least 10 minutes followed by a chilled water bath (4°C) for at least two minutes.

The Example below exemplifies the invention.

EXAMPLE

Detection of HIV I

A. Amplification of Target Nucleic
Acid by PCR

The procedure as described in U.S. Patent 4,683,202 and in a product bulletin for GeneAmp DNA Amplification Reagent Kit (#N801-0043) can be followed utilizing the following specific conditions and reagents. Two sequences of the HIV I genome can be selected to be amplified. The first is a 103-nucleotide base sequence located within the GAG p17 region of HIV I, incorporated into a plasmid (the plasmid incorporating most of the HIV I genome is designated pBH10-R3), and can be amplified using primers A and B as shown below:

5'..ATAACTTCGTATAGCATAACATTATACGAAGTTATTGGGCAAGCAGGGAGCTAGG
..3'

Primer A

5'..ATAACTTCGTATAGCATAACATTATACGAAGTTATTCTGAAGGGATGGTTGTACG
..3'

Primer B

The second is a 160-base region also located within the GAG p17 region of HIV I, incorporated into a plasmid (the plasmid incorporating most of the HIV I genome is

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designated pBH10-R3), and can be amplified using Primers C and D as shown below:

5'..ATAACTTCGTATAGCATAACATTATACGAAGTTATTTCCCTCAGACCCTTTTAGTC.
.3'

5

Primer C

5'..ATAACTTCGTATAGCATAACATTATACGAAGTTATTGGCGTACTCACCAGTCGC
CT..3'

Primer D

10 Aliquots of serial dilutions (1×10^7 , 1×10^6 ,
 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , and zero copies)
of plasmid pBH10-R3 can be amplified using PCR. Each
aliquot can be combined with a buffer 200 μ M in each of
dATP, dTTP, dCTP, and dGTP, 1.0 μ M in each of Primers A,
B, C, and D, and containing 1 μ g of human placental
15 DNA/reaction and 2.5 units of a DNA polymerase, in a
total reaction volume of 100 μ l.

Each reaction mixture can then be temperature
cycled as described in the product bulletin thirty (30)
times.

20 This process is expected to result in the estimated
increase in the number of target molecules by 1×10^5 to
 1×10^6 .

The products of the amplification reactions can be
placed onto a 6% acrylamide gel run under standard
25 conditions. After electrophoresis, the gel can be
soaked in a 10 μ g/ml solution of ethidium bromide in 10
mM Tris, pH 7.0, for 15 minutes. The gel can then be
rinsed in 10 mM Tris, pH 7.0, and the resulting product
bands can be detected and/or measured by irradiating the
30 gel at 302 nm and visualizing the fluorescent bands
produced.

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CLAIMS

1. A nucleic acid assay for the detection and/or measurement of a preselected nucleic acid sequence in a sample suspected of including a nucleic acid containing said preselected sequence comprises the steps of:
- (A) rendering the target nucleic acid single-stranded;
- (B) amplifying two specific nucleic acid sequences contained within the preselected nucleic acid sequence, said specific nucleic acid sequences being positioned such that when either sequence is amplified under amplification conditions, the extension product of either sequence cannot serve as a template for the synthesis of the other sequence, by:
- (1) treating the strands with two sets of two oligonucleotide primers, one set for each different specific sequence being amplified, under conditions such that for each different sequence being amplified an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said sets of primers are selected so as to be sufficiently complementary to the different strands of each specific sequence to hybridize therewith such that the extension products synthesized from one primer from each of the two sets of primers, when separated from their respective complements, can serve as templates for the synthesis of the extension products of the other primer from each

of the two sets of primers; wherein one of the primers of each set of primers contains a LoxP sequence at its 5'-end;

5

(2) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

10

(3) treating the single-stranded molecules generated from step (2) with the two sets of primers of step (1) under conditions that primer extension products are synthesized using each of the single strands produced in step (2) as templates; and

15

(4) repeating steps (2) and (3) to produce sufficient primer extension products for detection and/or measurement;

20

(C) treating the products of the two separate amplification reactions by the recombinase enzyme Cre; and

(D) detecting and/or measuring that product of step (C) resulting from the two separate amplification reactions.

25

INTERNATIONAL SEARCH REPORT

International Application No

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I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/68; C12P 19/34; C01N 33/52 U.S.Cl.: 435/6, 435/91		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/6; 435/91	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
COMPUTER SEARCH OF APS AND CAS DATABASES		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁸	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	Journal of Molecular Biology, volume 187, issued 1986 pages 197-212; Sternberg et al.: "Bacteriophage P1 cre Gene and its Regulatory Region, "Evidence for Multiple Promoters and Regulation by DNA Methylation"	1
A	EP, A, 0,246,864 (Imperial Chemical Industries) Carr issued 25 NOVEMBER 1987, see abstract.	1
A	US, A, 4,683,202 (MULLIS) 28 JULY 1987, See claim 17.	1
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ * Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
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				US	United States of America

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US90/01535

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U.S.Cl.: 435/6, 435/91

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Minimum Documentation Searched *

Classification System

Classification Symbols

U.S. 435/6; 435/91

Documentation Searched other than Minimum Documentation
to the extent that such documents are included in the fields searched *

COMPUTER SEARCH OF APS AND CAS DATABASES

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IV. CERTIFICATION

Date of the Actual Completion of the International Search :

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